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Technical Note

Establishing species-specific sexing markers suitable for non-invasive samples of species lacking genomic resources: an example using the highly endangered common hamster *Cricetus cricetus*

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Abstract

Here we present an approach to establish species-specific genetic markers for sex identification suitable for non-invasive samples. Such markers are not yet available for the endangered common hamster (*Cricetus cricetus*) because of the lack of genomic resources. Using Y chromosome conserved anchored tagged sequences (YCATS) exonic primers, we obtained Y-chromosomal sequences from hamsters and sympatric rodent species. From this, we designed hamster-specific primers targeting two short Y-chromosomal intron fragments and included them in microsatellite multiplex reactions, using autosomal loci also as amplification controls. The method yielded highly consistent results. The approach can be easily applied to development of sex markers in species for which there are no genome sequences available and thus aid conservation genetics efforts.

Reliable sex identification is key for understanding mating systems, structure and dispersal strategies in natural populations. Genetic Y-chromosome markers are relatively straightforward to develop for species where genomic data from males are available, such as for prominent taxa like carnivores and elephants (Ahlering et al. 2011; Bidon et al. 2013). Genome sequences are, however, rarely available for many non-model or most endangered species (Greminger et al. 2010).

Here we present how to develop sensitive, species-specific genetic sex markers that are suitable for non-invasive samples. Genotyping non-invasive samples has

become a standard tool in wildlife genetics (Beja-Pereira et al. 2009). Our assay design comprises a double Y-chromosomal amplification as described in Bidon et al. (2013), which is essential for non-invasive samples with high allelic dropout and thus mimic a female. We developed the Y chromosome marker for the endangered common hamster (*C. cricetus*), a species that shows an ongoing rapid decline with regional extinctions across the entire range (La Haye et al. 2012; Meinig et al. 2014; O'Brien 2015).

We obtained Y-chromosome sequences for *C. cricetus* and in five sympatric rodent species using Y chromosome conserved anchored tagged sequences (YCATS) primers (Hellborg and Ellegren 2003). These exon-located primers amplify Y chromosome loci in a wide range in mammals, and span introns which exhibit high inter-, but only relatively low intraspecific divergence (Greminger et al. 2010). To design hamster-specific primers, we selected and amplified the loci *SMCY17* and *DBY8*, due to published amplification success and product sizes of ~200 bp in several species (see Hellborg and Ellegren 2003).

DNA was extracted from male and female tissue of *C. cricetus* and five mammal species—bank vole (*Myodes glareolus*), yellow-necked mouse (*Apodemus flavicollis*), house mouse (*Mus musculus*), brown rat (*Rattus norvegicus*) and common shrew (*Sorex araneus*)—using Blood&Tissue Kits (Qiagen, Hilden Germany). YCATS fragments were amplified using a 15 µl PCR containing 3 µl 10 pmol/µl DNA extract with 0.2 U/µl BSA, 3 µM MgCl, 1× Polymerase Buffer, 0.2 µM dNTPs, 0.3 µM each primer, and 0.66 U/µl TAQ Polymerase. PCR reactions were performed in a Biometra T1Plus thermocycler (Analytik Jena), with 5 min at 95 °C followed by 35 cycles for 30 s at 95 °C, 90 s at 58 °C and 60 s at 72 °C and 15 min final elongation at 72 °C, followed by an ExoSAP-IT (Amersham Bioscience®) clean-up. Sequencing was performed in both directions using a 3730xls Analyzer (Applied Biosystems). Based on sequence alignment using Geneious 7.0.6 (obtained sequences NCBI: KY358227–KY358235), two new hamster-specific primer pairs for each locus were designed using Primer 3 (Untergrasser et al. 2012) (Table 1; Alignment in suppl.1).

Table 1: Simple multiplex assay. Locus, primer sequences and fragment sizes of designed Y-chromosomal markers and an autosomal microsatellite

Genomic location	Locus name	Primer sequence (5'-3')	Fragment size [bp]*
Y-linked	*DBY8-Cricri*	F: GAAAAGTAAGCATAGCATTTAG	106
		R: CGGACTCTAGACCTGTAA	
Y-linked	*SMCY17-Cricri*	F: CTCAAGGAGCCGACAATAT	94
		R: GACCAGGGAAAAAAGTATGTAG	
Autosomal	Ccrµ10	F: TATATGCACATCATGTCACG	147–165
		R: GGTCTTAAGAATCAGGTGTG	

* fragment sizes are given as determined from the sequences used for primer design, see Appendix 1.

The new markers, SMCY17-Cricri and DBY8-Cricri, were tested in a simple multiplex PCR including 2 µl DNA extract, 1xQiagen® Multiplex Mix with a concentration of 0.2 µM for each primer. Primers for the hamster-specific microsatellite Crcri10 (147-165bp; Reiners et al. 2014) were added as amplification control. Finally, the sex markers were also added to a complex multiplex PCR including six microsatellite loci (Reiners et al. 2014). Thermocycling started with 95 °C for 15 min, followed by eight cycles of 30 s at 94 °C, 90 s touchdown starting with 60 °C, lowering 1 °C per cycle, and 60 s at 72 °C. PCR proceeded with 27 cycles of 30 s at 94 °C, 90 s at 52 °C, and 60 s at 72 °C, with 30 min final elongation at 60 °C. Amplicons of the simple multiplex were loaded on 2.5% agarose gels to verify amplification (Fig. 1a). Allele sizing of the complex multiplex was performed on a 3730xls Analyser (ABI). Electropherograms were scored with GeneMarker 1.9 (Fig. 1b).

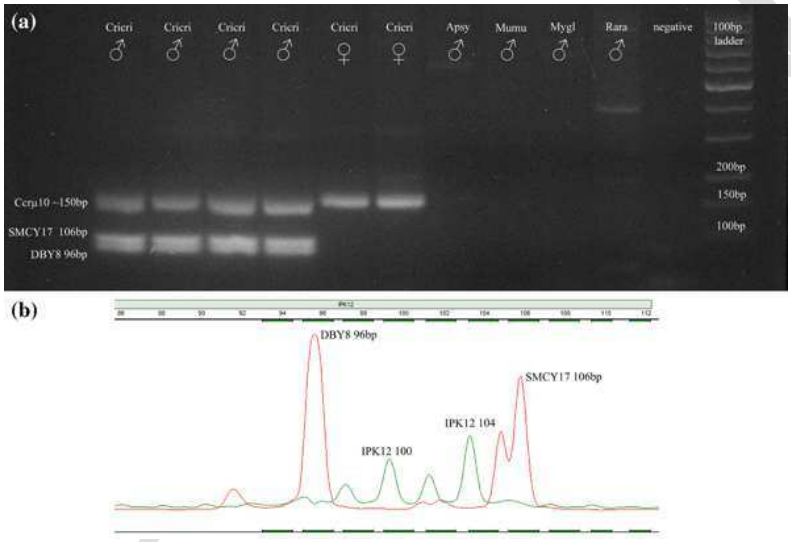

Figure 1: **a** Electrophoresis of the simple multiplex PCR products of SMCY17-Cricri and DBY8-Cricri, and the microsatellite Crcri10 as positive control. Tissue extracts of male and female common hamsters (*Cricri*) and four male non-target rodent species (Apsy = *Apodemus flavicollis*, Mumu = *Mus musculus*, Mygl = *Myodes glareolus*, Rara = *Rattus norvegicus*). **b** Electropherogram of the complex multiplex for a male hamster showing SMCY17-Cricri, DBY8-*Cricri* and microsatellite *IPK12* peaks.

The reliability of the essay was confirmed by three experiments. First, we verified that the simple multiplex gave expected results for male and female hamsters, while failed amplifying other rodents (Fig. 1a). Second, we analysed 179 ear-tissue extracts from live-trapped hamsters. All reactions were replicated three times to quantify genotyping errors. For adult hamsters (n = 71), our assay confirmed the sex determined by morphological inspection. Discrepancies were found for 7.8% (5 of 64) subadult and 15.8% (6 of 38) juvenile hamsters, suggesting that the field method is error-prone. Third, we analyzed 116 hamster hair samples collected using non-invasive trapping (Reiners et al. 2011). DNA was extracted using the Qiagen® Investigator Kit. Allelic dropout rate of the sex markers was only 2.3% across replicates of male samples, compared to 3.3% for microsatellites.

The results show that our new sexing method using YCATS intronic primers is reliable and that molecular sexing is

superior to sexing in the field, where juvenile sex was misidentified. Furthermore, this study highlights that using YCATS allows the establishment of species-specific sex markers. Past successful approaches in wolves (Sastre et al. 2005), canids (Seddon 2005), wolverines (Hedmark et al. 2004), and large felids (Sugimoto et al. 2006), demonstrate the high potential of this approach.

Supplementary material

The article contains supplementary material.

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